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CENTRAL NERVOUS MONOAMINES AND HORMONE INDUCED ESTRUS BEHAVIOUR IN THE SPAYED RAT

BY

BENGT J. MEYERSON

UPPSALA 1964

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Det arbete, som härmed framlägges, omfattar följande delarbeten:

1. *Meyerson, B. J.*, The effect of neuropharmacological agents on hormone-activated estrus behaviour in ovariectomised rats. Arch. int. Pharmacodyn. 1964, 150, 4–33.
2. *Meyerson, B. J.*, Estrus behaviour in spayed rats after estrogen or progesterone treatment in combination with reserpine or tetrabenazine. Psychopharmacologia. 1964, 6, 210—218.
3. *Meyerson, B. J.*, Central nervous monoamines and hormone induced estrus behaviour in the spayed rat. Acta physiol. scand. 1964, 63, Suppl. 241.

Utöver redogörelse för resultaten av nya undersökningar, omfattar arbete nr 3 en sammanfattning av avhandlingens problemställning samt vunna egna forskningsresultat.

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FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF UPPSALA, SWEDEN
(HEAD: PROF. DR. ERNST BÁRÁNY).

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Introduction

It is well known that the estrus behaviour in female mammals, with cyclic periods of heat, is dependent on gonadic hormones. The relationship between estrus behaviour and estrogen in the female rat was studied thoroughly by Hemmingsen (1932). He showed, in agreement with earlier similar experiments of Allen (1924), that ovariectomy brought about almost immediate loss of estrus behaviour, while the accepted criteria of heat could be produced by injection of estrin. Several authors have described the same effects in other animals and this has been reviewed by Beach in 'Hormones and Behaviour' (1948).

There is reason to assume that the estrus behaviour is activated by a direct effect of the estrogen on structures within the hypothalamus. Harris et al. (1958) showed that stilbestrol dibutyrate implantation in the posterior hypothalamus of the spayed cat resulted in complete estrus behaviour in spite of no peripheral estrus manifestations. Estradiol implantation in the anterior hypothalamus activated heat response in spayed rats, while implants within other parts of the hypothalamus had no such effect (Lisk, 1962).

The significance of the hypothalamus for female estrus behaviour is also indicated by the results from lesion experiments, and electric recording which are reviewed by Sawyer (1960).

A possible relationship between cerebral monoamines and this hormone-activated behaviour was investigated in view of the accumulating evidence that monoamines are involved in transmitter activity within the central nervous system.

The estrus behaviour in ovariectomised rats is normally activated by progesterone after pretreatment with estrogen. Boling and Blandau found (1939) that in rats the female heat response is far more effectively activated by this combination of the two hormones than by estrogen treatment alone. It has recently been shown that amine depletion by reserpine or tetrabenazine (Meyerson, 1964 b) could be substituted for progesterone in the estrogen-progesterone treatment. The estrogen is still necessary. Moreover, increase of cerebral monoamine levels achieved by monoamine oxidase inhibitors reduced the estrogen-progesterone activated heat response (Meyerson, 1964 a). This led to the suggestion that monoaminergic central nervous pathways exist which mediate heat inhibition.

The purpose of the present investigation was to investigate further the relation between the hormone-activated estrus behaviour and the central nervous catecholamine and serotonin levels.

Methods

A. Experimental animals

Ovariectomised albino Sprague-Dawley rats, weighing 180—300 grams, were used. They were fed commercial rat pellets (Anticimex 210, Norrviken, Sweden) and tap water ad libitum. The ovariectomy was permormed 2—3 weeks before the animals were used in experiments; the absence of estrus cycle checked by taking vaginal smears during a period of 6 days.

B. Estrus behaviour tests

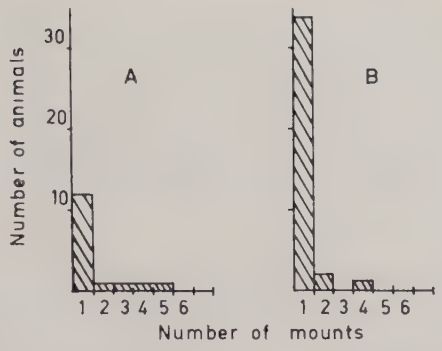
The display of lordosis response when mounted by a male was used as an indicator of estrus behaviour. Other behaviour manifestations associated with heat such as acceptance of the male, the female darting movements or ear wiggling were found difficult to estimate consistently at submaximal dose levels.

The method used follows that earlier described (Meyerson, 1964 a). The estrus behaviour was activated by injection of estradiol benzoate and progesterone, in that order, 48 hours apart. The animals were kept under reversed day-night rhythm (light from 9 p. m.—9 a. m.). Progesterone was administered at 8 a. m.; the optimal response is achieved 6—8 hours later.

The behaviour of each female was tested with 2 different vigorous males for a period of 1 minute each. It was earlier described (Meyerson 1964 a) that sedation of the females reduced the eagerness of the male to copulate. Besides using selected active males throughout the investigation this problem was overcome by the following maneuver: a highly receptive female, treated only with hormones, was introduced into the observation cage for a few moments prior to the sedated female.

Each female was offered at least five mounts per test; the males were immediately changed if exhausted, to ensure that this amount was reached. The frequency of mounts during the 2 minute tests only exceptionally exceeded 8—10 mounts. The test was interrupted as soon as the female had displayed a clearcut lordosis response.

Fig. 1 shows the number of mounts necessary to elicit the lordosis response at two dose levels of estradiol benzoate, 2.5 and 25 μg/kg followed 48 hours later by 0.4 mg/animal progesterone. Most animals responded to the first mount.



Treatment		Estrus response %	Number of animals tested
At 0 hrs	At 48 hrs		
A.E, 2.5 µg/kg	progesterone, 0.4 mg/anim	27	60
B.E, 25 µg/kg	progesterone, 0.4 mg/anim.	88	42

FIGURE 1. Frequency distribution of the number of mounts necessary to elicit lordosis reflex in estradiol (E) + progesterone treated spayed rats.
The animals were tested 8 hours after the progesterone treatment. Six mounts were offered.

C. Injected materials

Estradiol benzoate and progesterone were dissolved in olive oil and injected in a volume of 0.20—0.30 ml.

Neuropharmacological compounds used were: Reserpine (Serpasil®, CIBA ampoules 2.5 mg/ml), Mo 911 (N-methyl-N-benzyl-2-propynylamine hydrochloride: Pargyline®, Abbot Lab.), 5-HTP(DL-5-hydroxytryptophane, Sandoz), DOPA (DL-dihydroxyphenylalanine, Hoffman-La Roche), DOPS (DL-threo-3,4-dihydroxyphenylserine, Hässle), H 22/54 (α-propyl-dopacetamid, Hässle).

The reserpine blank used consisted of 10 % polyethyleneglycol 400, 1 % ethyl alcohol, 0.2 % EDTA and 0.1 % citric acid. 5-HTP was dissolved in 0.01 N HCl and the solution diluted with 9 volumes of saline. The other substances were dissolved in saline.

Doses mentioned in the text and tables always refer to the forms of the compounds stated above. All injections were subcutaneous unless otherwise stated.

D. Estimation of catecholamines and serotonin

The animals were sacrificed by decapitation and the brains immediately removed. Dissections were performed on ice-cold ceramic tiles.

Whole brains were cut into pieces and immediately put into the homogenizer containing ice-cold 0.4 N perchloric acid, EDTA and ascorbic acid (see below).

In experiments where further dissection was carried out this was done in two different ways which in the following will be referred to as Method A and Method B. Cerebellum, pons and medulla oblongata were excluded in both methods. Fig. 2 was constructed to facilitate the description of the dissection.

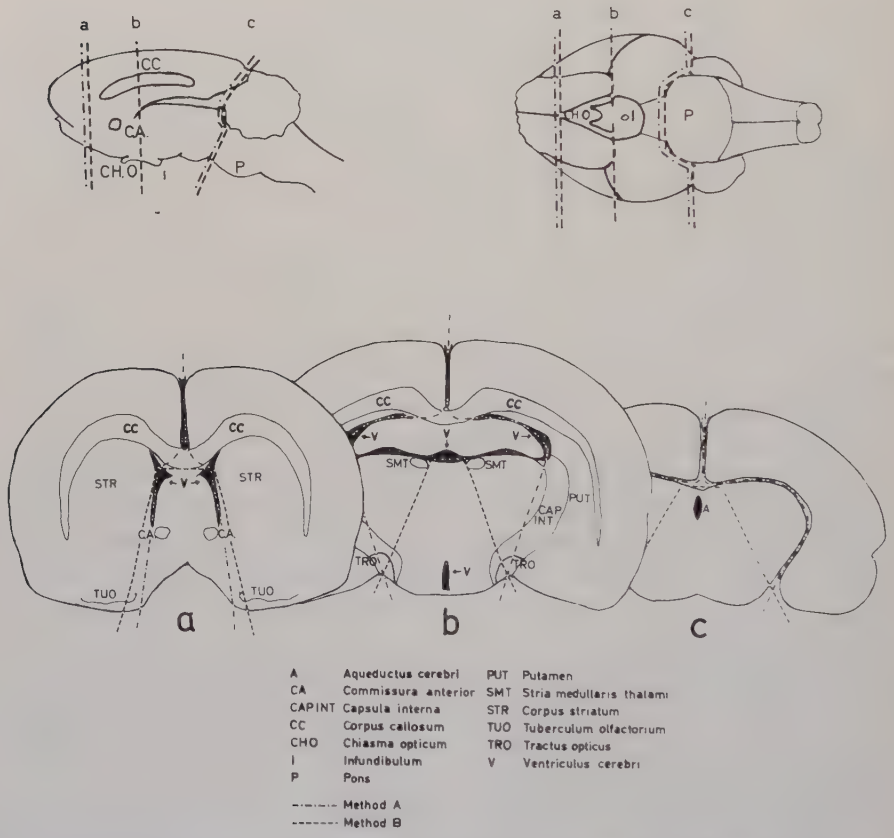


FIGURE 2. Dissection procedure used in the amine assay experiments. The lower pictures show the section surfaces of the transverse sections a, b, and c.

METHOD A. In the first place the brain was placed upside down and two transverse sections were made one immediately anterior to pons (Section c in Fig. 2; the separation of the two lines is only for clarity), the second anterior to the optic chiasm in the apex of the angle formed underneath the brain by the two olfactory tubercles (Section a in Fig. 2). The former section was made perpendicular to the longitudinal axis of the medulla oblongata.

From the upper side of the brain the lateral ventricles were reached via the sulcus centralis (see point-dash line). The ventricles were followed bluntly downwards to their inferiolateral corner from where sharp dissection was made to the furrow underneath the brain formed by the borderline between the hemispheres and the diencephalon — mesencephalon region.

The portion within the point-dash line in Figure 2, bounded anteriorally by the transverse section a, and posteriorally by c, constituted one fraction (Fraction A 1) and the rest of the tissue was collected into another fraction (Fraction A 2) for separate assay.

METHOD B. In addition to the above mentioned transverse sections a and c, a third was made half way between the optic chiasm and the infundibular stalk (Section b in Fig. 2). The slices of tissue between a and b and between b and c were turned with the section surface b upwards and a vertical cut was made between the tractus opticus and striae medullares, structures which are both easily recognised macroscopically (see dash line).

Three fractions were taken for separate assay. Fraction B 1 was the portion within the dash line between the transverse sections a and b, fraction B 2 that within the dash line between the transverse sections b and c. The remaining brain tissue was collected into one portion, Fraction B 3.

The purpose of this dissection was to cut out the regions expected to be relevant to estrus behaviour and monoamines (A1, B1, B2).

Fraction A1 comprised the diencephalon, mesencephalon and a minor part of the capsula interna.

Fractions B1 and B2 comprised the medial parts of the thalamus, the hypothalamus and partly the mesencephalon, mainly the rostral part.

By means of the fluorescence method for histochemical demonstration of monoamines (Falck, 1962) the occurrence of monoamine containing neurons in the brain was recently demonstrated. It was shown that the mesencephalon mainly contained cell bodies while fine varicose fibres assumed to be the terminal parts of axons were demonstrated within the hypothalamus (Carlsson, Falck and Hillarp, 1962; Dahlström and Fuxe, 1964).

Mean values of tissue-weight (g) ± S. D. for analyses (5 brains pooled) were:

Fraction	Tissue weight	Number of replicates
A 1	1.76 ± 0.13	(16)
A 2	5.09 ± 0.23[1]	(16)
B 1	0.39 ± 0.08	(14)
B 2	0.56 ± 0.03	(14)
B 3	5.16 ± 0.39[1]	(13)

Catecholamines

The analyses of catecholamines were performed by the method of Bertler, Carlsson and Rosengren (1958), with slight modifications described in the following.

Adrenaline was estimated throughout the investigation. The amounts achieved were, however, never great enough for specific assay. An account of these results will therefore not be given.

The tissue was homogenized by means of a teflon homogenizer. (Thomas Philadelphia, 4288 B) in cold 0.4 N perchloric acid, about 4 ml/g tissue, containing 1 mg/ml disodiumethylendiaminetetraacetic acid (EDTA) and 0.5 mg/ml ascorbic acid.

Addition of these metal complexing and antioxidant agents to the perchloric acid was found to improve and stabilize recoveries of dopamine especially. The remaining extraction was carried out according to the original method. One reextraction was done (2 ml/g tissue). The combined filtrates were kept at — 19° C overnight, only exceptionally for longer time (maximally 7 days).

After the extract had been thawed the pH was adjusted to 6.4—6.5 with 5 N potassium carbonate (checked by glass electrode).

For purification of the extract a column of Dowex 50 W, X—4, 200—400 mesh, 5.5 × 35 mm, in the sodium form was used. The treatment of the resin was according to the original method except that to the sodium acetate-acetic acid buffer was added 0.1 % EDTA (Häggendahl, 1962). Elution was performed with 11 ml of 1 N HCl followed by 11 ml 1.5 N HCl. The first 3 ml were not collected, the next 8 ml were taken for noradrenaline and adrenaline assay and the remaining 11 ml for dopamine assay.

In order to increase the concentration of the fluorophore the amount of solutions added to the sample (2 ml) for noradrenaline and adrenaline

[1] The homogenate was divided into equal parts.

assay, was reduced to one half of that described by Bertler et al. and the amount of water added was adjusted to yield a final volume of 5 ml.

Dopamine was determined according to Carlsson and Waldeck (1958) with the modification that the amount of 0.02 N iodine solution was increased to 0.1 ml and the final pH was adjusted by 1.6 ml 5 N acetic acid (Carlsson and Lindqvist, 1962).

Serotonin

The tissue was homogenized in 2—3 ml/g 0.1 N HCl and the entire sample was immediately extracted according to the method of Bogdanski et al. (1956) with the modification of the quantities of reagents given by Udenfriend (1958). However, no borate buffer wash was done except when the animals had been treated with 5-hydroxytryptophane. Preexperimental results revealed the same serotonin values with and without this wash.

*

The fluorescence was read in an Aminco Bowman spectrophotofluorometer equipped with an Osram XBO lamp. After rearrangement to corresponding fluorophores the activation and fluoroscence maxima for authentic noradrenaline (L-noradrenaline D-bitartrate monohydrate, N. V. Philips-Duphar, Amsterdam) dopamine (3-hydroxy-tyramine-hydrochloride, Hoffman-La Roche, Basle) adrenaline (L-adrenaline bitartrate, Rhône Poulenc, Paris) and serotonin (5-hydroxytryptamine creatinine sulfate, Hoffman-La Roche, Basle) were identical with those obtained from the brain eluate.

The fluorescence intensities were read at (uncorrected instrumental values, activation wave length/fluorescence wave length, both in mμ): serotonin 295/545, dopamine 325/275, noradrenaline, adrenaline 400/515 and 450/515.

The fluorescence of the samples was compared with a pure standard (a standard treated in the same way as the 2 ml sample of the catecholamine eluate). For serotonin the standard was treated identically with the final acid extract. There was good agreement between the pure catecholamine standards and an internal standard (a standard added to a tissue eluate). No such comparison was made with serotonin.

Known amounts of catecholamines and serotonin were regularly added to one half of a divided homogenate. Recoveries ranged from 70—100 % with mean values about 85 %. Whole brain values are evident from Table 1. The values of catecholamines and serotonin are expressed as free base throughout the investigation.

TABLE 1.
Levels of serotonin, noradrenaline and dopamine, in whole brains of ovariectomised Sprague-Dawley rats (250—300 g). Two brains were pooled. Number of replicates = 7. The values are means ± standard errors of the mean, expressed in μg/g brain.

SEROTONIN	0.55 ± 0.02
NORADRENALINE	0.55 ± 0.01
DOPAMINE	0.75 ± 0.03

The analyses of tissue from fraction B 2 yielded rather small amounts of dopamine, the fluorescence of the sample taken for estimation was at the lower limit of measurability. However, in no case was the read fluorescence less than two times the tissue blank value.

Statistical treatment of amine analyses

Controls and experimentals were run at the same time unless otherwise stated. Whenever the significance of data was analyzed statistically, Student's t-distribution was used.

Percentage increases and decreases were calculated as:

$$\frac{Expt. - control}{control} \times 100$$

The control values were derived from the mean of two estimates of single brains for catecholamines and two for serotonin, run at the same time as the experimentals; the values are given in the figure legends. These controls were spayed and got the same hormone treatment as experimentals taken for amine assay. The control animals are not included in the number of animals reported in the text or shown in the figures.

Separate controls were run with each experiment because the variability between experiments was much larger than within an experiment.

*

Even if the same treatment of a control group appears in different tables or figures, the control experiments were always repeated for each experiment.

Results

I. The influence of increased cerebral levels of serotonin, dopamine and noradrenaline on estrogen-progesterone activated estrus behaviour

It has recently been shown that the estrogen-progesterone activated heat response is reduced by monoamine oxidase inhibitors (Meyerson 1964 a). The purpose of the present experiments was to study the relationship between this inhibitory effect on the heat response and increased cerebral levels of noradrenaline, dopamine and serotonin.

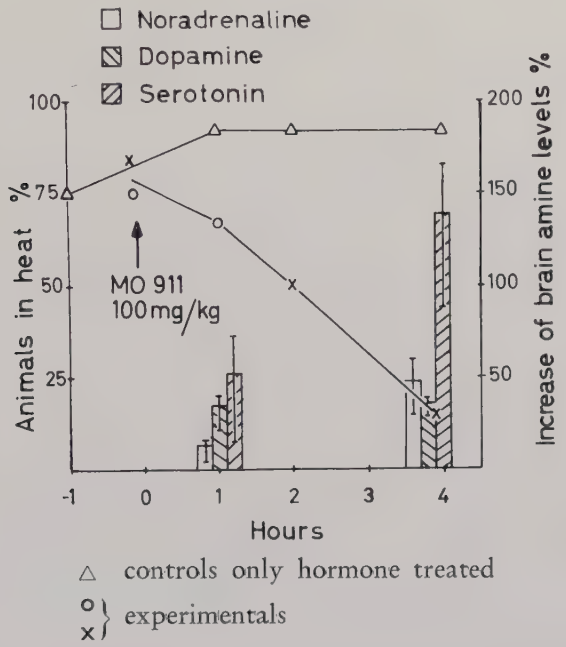


FIGURE 3. The effect of monoamine oxidase inhibition by Mo 911 on estradiol-progesterone activated estrus behaviour and cerebral monoamines in spayed rats.
Mo 911 was injected 4 hours after the progesterone administration. The curves describe the percentage of animals in heat. Each column represents the mean of six amine determinations on single brains. Vertical lines represent range. Control values (μg/g): serotonin 0.63. noradrenaline 0.62, dopamine 0.80. The same control values were used in the 1 and 4 hour experiment. The timespan between tests for estrus behaviour and sacrifice for amine analyses was 10—15 minutes.

The substance Mo 911 which was used in this experiment has been described as a potent irreversible inhibitor more specific for monoamine oxidase than the hydrazines (Everett, Wiegand and Rinaldi 1963, see also the subsequent discussion by Zeller).

The estrus behaviour was activated by estradiol benzoate, 25 μg/kg followed 48 hours later by progesterone, 0.4 mg/animal.

A relationship between the accumulation of cerebral serotonin and noradrenaline and decreased heat response is evident from Fig. 3. Only the value of dopamine 1 hour after Mo 911 was almost the same as that at 4 hours, a 40 % increase over the controls.

The experiment included 36 animals, 12 only hormone treated (controls Fig. 3 — △ —); 24 got 100 mg/kg Mo 911 and 12 of these latter were sacrificed for amine analyses 1 hour (Fig. 3 — o —) and the other 12 animals 4 hours (Fig. 3— x —) after the Mo 911 treatment.

The change of the general behaviour of estrogen-progesterone treated spayed rats after Mo 911 treatment was described in an earlier investigation (Meyerson, 1964 a). The main features 4 hours after injection were that the animals strain when handled and become "slightly intractable".

Manifestations of heat other than the lordosis reflex such as darting movements and ear wiggling which were seen in most of these animals before the Mo 911 was administered, gradually disappeared and were completely absent at 4 hours after the injection. At this time the rejecting kicks with the hind legs when mounted were very frequently seen.

In order to study the effect of acute selective increases of dopamine, noradrenaline and serotonin, the corresponding amine precursor DOPA, DOPS and 5-HTP were administered 15 minutes after 100 mg/kg Mo 911 (Fig. 4). DOPA is a wellknown precursor of dopamine, 5-HTP of serotonin, while DOPS is decarboxylated to noradrenaline (see Carlsson, 1964).

Tests for heat response were performed one hour after the injection of Mo 911. Fig. 4 shows that even greatly increased dopamine and noradrenaline levels did not decrease the heat response, while there was complete absence of estrus behaviour in animals which had recieved 3 mg/kg 5-HTP in combination with the Mo 911 treatment and showed increased serotonin levels.

One hour after treatment with Mo 911 in combination with DOPA or 5-HTP, the general behaviour was hardly changed. Surprisingly the Mo 911 + DOPS treated animals never showed any excitement, but rather decreased activity; they sat inactive in their cages but moved about in the observation cage when tested for heat response, with normal or slightly reduced exploring behaviour. The change of the behaviour seemed not to

be in proportion to the fact that the combination turned out to be toxic; 4 out of 10 animals were dead the following day.

The estrus behaviour tests were continued with 10 of the Mo 911 + DOPS treated animals. Two hours after Mo 911 had been administered 7/10 showed positive heat response and at 4 hours 2/10. These are the same values as obtained by Mo 911 treatment alone (Fig. 3). Thus, adding DOPS to the Mo 911 treatment neither increased nor decreased the inhibition of the heat response obtained by Mo 911 administration. This was true also when DOPA was administered instead of DOPS in similar experiments (Meyerson 1964 a).

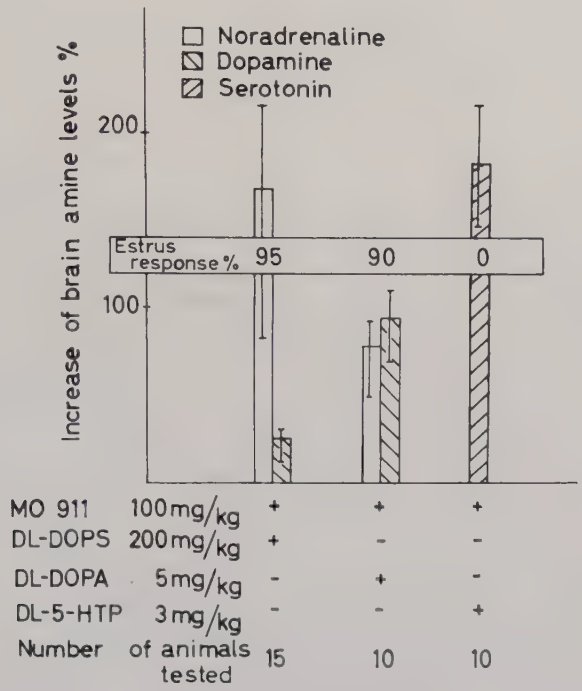


FIGURE 4. The effect of monoamine oxidase inhibition by Mo 911 in combination with the amine precursors DOPS, DOPA and 5-HTP on estradiol-progesterone activated estrus behaviour in spayed rats. Mo 911 was injected 4 hours and the precursors 4 1/4 hours after progesterone administration. The estrus behaviour was tested one hour after Mo 911 treatment. With these doses 90—95 % of only hormone treated animals responded with heat in control experiments. The timespan between test and sacrifice for amine analyses was 10 min. Each column represents the mean of five determinations on single brains. Vertical lines represents range. Control values (μg/g): serotonin 0.51, noradrenaline 0.45, dopamine 0.78.

The substance H 22/54 has been described as a potent catechol-O-methyl transferase inhibitor, which also blocks the biosynthesis of catecholamines and serotonin, probably by inhibiting the enzymatic hydroxylation of L-phenylalanine, L-tyrosine and L-tryptophane (Carlsson, Corrodi and Waldeck, 1963). The same authors showed that the increase of cerebral serotonin levels after monoamine oxidase inhibition is prevented by administering H 22/54.

In the present experiments H 22/54 was administered half an hour before and one hour after the Mo 911 injection; 2 × 300 mg/kg reduced the accumulation of serotonin as can be seen from Fig. 5 compared with Fig. 3.

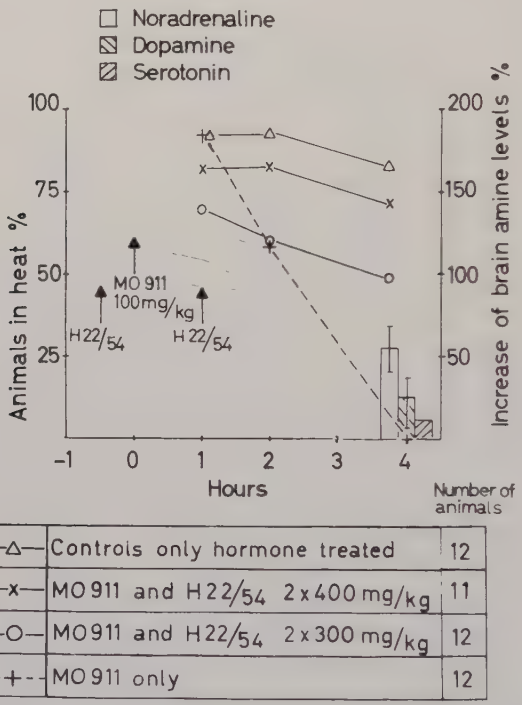


FIGURE 5. The effect of monoamine oxidase inhibition by Mo 911 in combination with the substance H 22/54, 2 × 300 mg/kg on estradiol-progesterone activated estrus behaviour in spayed rats. Mo 911 was injected 4 hours after the progesterone administration. The sacrifice for amine analyses was made 4 hours after Mo 911 treatment. The curves describe the percentage of animals in heat. Each column represents the mean of four determinations on single brains. Vertical lines represent range. Control values (μg/g): serotonin 0.55, noradrenaline 0.64, dopamine 0.73. The serotonin values: — 36, — 36, + 42, + 71 % change from control.

Dopamine and noradrenaline levels were similar to those obtained by Mo 911 alone. The inhibitory effect of Mo 911 on estrus behaviour was clearly reduced.

A single dose of H 22/54, 500 mg/kg, 30 minutes before Mo 911 administration in otherwise analogous experiment also tended to protect the estrus behaviour from inhibition by Mo 911. The figures were: 8/9 animals responded at 1 hour after Mo 911 treatment and 6/9 at 2 and 4 hours.

The change in general behaviour after Mo 911 treatment was not seen in animals which had also been given H 22/54. In contrast to what was seen after Mo 911 alone, several animals showed darting movements and ear wiggling when H 22/54 was administered as well. However, animals which did not display a positive heat response 4 hours after the Mo 911 administration still displayed the vigorous kicking with the hind legs when mounted, indicating that H 22/54 did not prevent these signs of sexual unreceptivity.

II. Estrus behaviour after estradiol-reserpine treatment

Normally the estrus behaviour is activated by estrogen in combination with progesterone. Estrus response after estrogen treatment alone requires very large doses of the hormone (more than 100 μg/kg, Meyerson 1964 b). In a recent investigation it was found that reserpine treatment could replace the progesterone administration (Meyerson, 1964 b).

The relationship between reserpine-elicited estrus behaviour and the degree of cerebral monoamine depletion after reserpine treatment was studied by taking advantage of the gradual onset of depletion after reserpine.

Two dose levels of estradiol were used, 2.5 and 10 μg/kg. The time from estradiol benzoate injection to estrus behaviour test was kept constant at 56 hours. Reserpine, 2 mg/kg was injected 1, 3 or 8 hours before the heat response test. Ten animals were treated with 10 μg/kg estradiol alone.

It is evident from Fig. 6, 7 and 8 that less depletion was necessary to obtain a response when the larger dose of estrogen was used. In the 10 μg/kg estradiol experiment almost all animals displayed estrus response when about 60 % of the cerebral amines had disappeared. Some animals responded already at 10—20 % depletion.

A comparison of the extent of noradrenaline, dopamine and serotonin depletion in whole brain after 1, 3 and 8 hours showed that the amines disappeared at very similar rates. Therefore, these curves do not show which one of the amines is the important one.

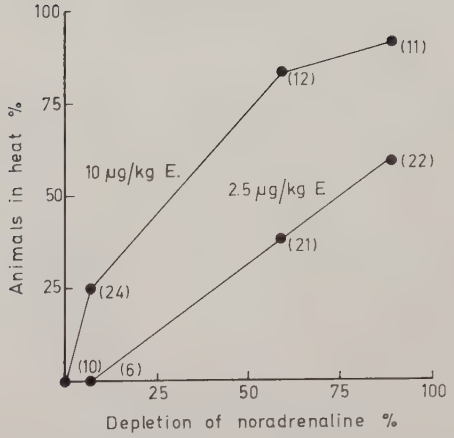


FIGURE 6. The relationship between the estrus response, elicited by reserpine, 2 mg/kg in estradiol treated (E) spayed rats, and the amine levels.
Each amine value (abscissa) is the mean of 3—4 estimations on single brains. Control values (μg/g): serotonin 0.56, noradrenaline 0.58, dopamine 0.73. The experimentals were taken from the 2.5 μg/kg E. experiments. The timespan between test and sacrifice for amine analyses was 10 min. Numbers within brackets show number of animals tested for estrus behaviour; the same animals appear in Figure 7 and 8.

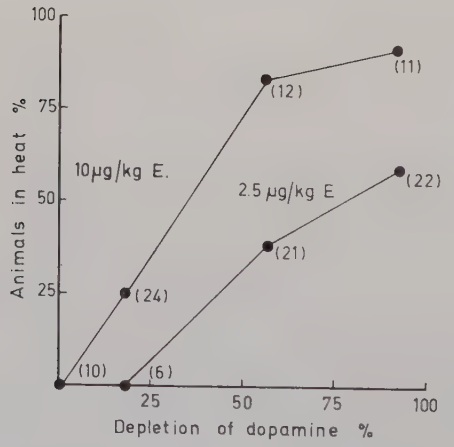


FIGURE 7. For text see Figure 6.

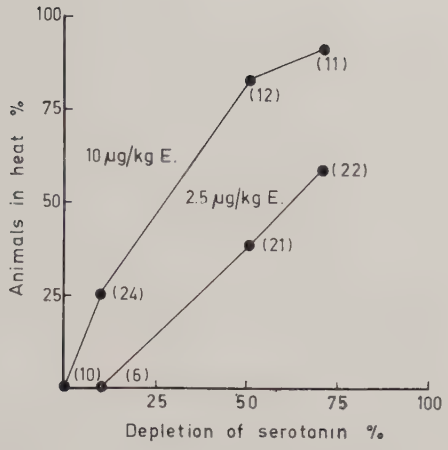
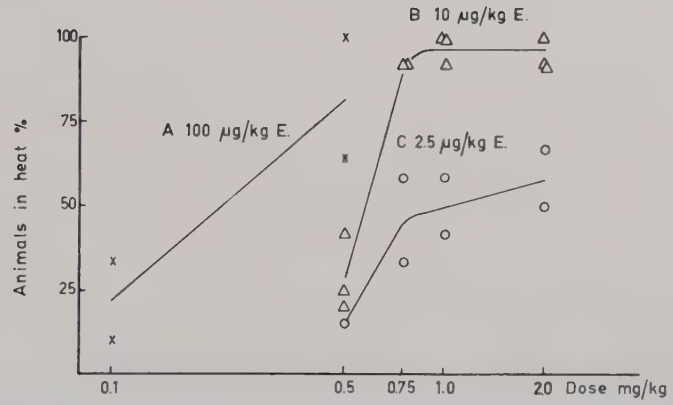


FIGURE 8. For text see Figure 6.



Number of animals tested	22	23			A	x
		34	24	36	32	B	△
		12	24	23	22	C	o

FIGURE 9. The relationship between the dose of reserpine and the percentage of estradiol (E) treated spayed rats responding with estrus behaviour.
Each point represents one run.
Reserpine was injected 48 hours after the administration of E. Tests for estrus behaviour were done 8 hours after the reserpine injection.

Four dose levels of reserpine were tested after pretreatment 48 hours earlier with 100, 10 or 2.5 μg/kg estradiol benzoate. Fig. 9 shows the heat response 8 hours after the reserpine administration. With the estrogen dose of 100 μg/kg which alone never causes heat response even 0.1 mg/kg reserpine was sufficient to bring 5 of 22 animals into heat. The data will be further interpreted in the Discussion. Motor activity was only very slightly reduced after 0.5 mg/kg reserpine. Three animals of 23 displayed ear wiggling and 1/23 showed obvious darting movements in the 100 μg/kg estradiol + 0.5 mg/kg reserpine experiment. Such behaviour patterns were observed occasionally even at lower dose levels of estrogen in combination with reserpine 0.5—0.75 mg/kg.

III. The effect of hormone treatment on the cerebral content of noradrenaline, dopamine and serotonin

The cerebral content of noradrenaline, dopamine and serotonin was investigated in spayed rats, treated with estradiol benzoate, 50 μg/kg and 48 hours later progesterone, 0.4 mg/animal. The animals were sacrificed 5 hours after the progesterone injection. At this time normally 100 % heat response is achieved with the hormone treatment used. Control animals had oil blank solutions instead of hormones.

The diencephalic-mesencephalic parts were dissected out according to method A for serotonin analyses and method B for catecholamine analyses. Method A was easier to repeat consistently due to the fact, that blunt dissection was possible to a great extent. However, method B was chosen for

TABLE 2.

The effect of estradiol-progesterone treatment on the cerebral content of serotonin in spayed rats.

Progesterone, 0.4 mg/animal was injected 48 hours after estradiol benzoate 50 μg/kg. The animals were sacrified 5 hours after the progesterone injection. Each replicate (5) consists of tissue from 5 brains. The values are means ± standard errors of the mean, expressed in μg/g brain.

DISSECTION METHOD A:	Hormone treated	Controls
Fraction 1	0.84 ± 0.05[1]	0.72 ± 0.04[1]
Fraction 2	0.48 ± 0.01	0.44 ± 0.02

[1] P of difference > 0.1.

TABLE 3.

The effect of estradiol-progesterone treatment on the cerebral content of noradrenaline (NA) and dopamine (DA) in spayed rats. Progesterone, 0.4 mg/animal was injected 48 hours after estradiol benzoate, 50 μg/kg. The animals were sacrificed 5 hours after the progesterone injection. Each replicate (4) consists of tissue from 5 pooled brains. The values are means ± standard errors of the mean, expressed in μg/g brain.

DISSECTION METHOD B:		Hormone treated	Controls
Fraction 1	NA	2.09 ± 0.22[1]	2.18 ± 0.20[1]
	DA	0.80 ± 0.16	0.97 ± 0.17
Fraction 2	NA	0.93 ± 0.18	0.98 ± 0.17
	DA	0.27 ± 0.05	0.33 ± 0.08
Fraction 3	NA	0.40 ± 0.05	0.45 ± 0.02
	DA	1.07 ± 0.17[2]	1.24 ± 0.06[2]
Fraction (1 + 2 + 3)	NA	0.55 ± 0.04	0.61 ± 0.004
	DA	0.98 ± 0.15[3]	1.13 ± 0.07[3]

P of difference: [1] > 0.5. [2] > 0.2. [3] > 0.2.

the catecholamine analyses as the dopamine rich regions nucleus caudatus and nucleus lentiformis were more completely separated by this method from the diencephalic part.

Noradrenaline in the hypothalamus has been shown to be concentrated in areas mainly within the anterior parts (Carlsson, Falk and Hillarp 1962). The amount of noradrenaline in the present investigation is in good agreement with that obtained by the same authors in the posterior and anterior-intermediate hypothalamus (1.2 resp. 1.9—2.3 μg/g).

It is evident from Table 2 and 3 that there is no statistically significant difference in the amount of assayed monoamines in the control and the combined hormone treated animals.

Nor are there statistically significant effects on the cerebral amines in opposite direction by estrogen and progesterone (Table 4, 5).

Good agreement between analyses of the two parts of divided homogenates and satisfactory recoveries make it probable that the rather large scatter of some of the catecholamine results was due to the difficulty in re-

TABLE 4.

The effect of progesterone or estradiol treatment on the cerebral content of serotonin in spayed rats. The animals were sacrificed 5 hours after 4 mg/animal progesterone resp. 53 hours after 50 μg/kg estradiol benzoate. Each replicate (3) consists of tissue from 5 brains. The values are means ± standard errors of the mean, expressed in μg/g brain.

DISSECTION METHOD A:	Progesterone treated	Estradiol treated
Fraction 1	0.78 ± 0.02	0.79 ± 0.04
Fraction 2	0.50 ± 0.00	0.47 ± 0.01

The two groups of treatment were not run at the same time.

TABLE 5.

The effect of progesterone or estradiol benzoate treatment on the cerebral content of noradrenaline (NA) and dopamine (DA) in spayed rats.
The animals were sacrificed 5 hours after 4 mg/animal progesterone resp. 53 hours after 50 μg/kg estradiol benzoate. Each replicate (3) consists of tissue from 5 brains. The values are means ± standard errors of the mean, expressed in μg/g brain.

DISSECTION METHOD B:		Progesterone treated	Estradiol treated
Fraction 1	NA	1.95 ± 0.05	1.97 ± 0.06
	DA	0.91 ± 0.10[1]	1.20 ± 0.12[1]
Fraction 2	NA	1.03 ± 0.05	1.05 ± 0.11
	DA	0.48 ± 0.05	0.37 ± 0.06
Fraction 3	NA	0.41 ± 0.05	0.42 ± 0.03
	DA	1.00 ± 0.06	1.00 ± 0.09
Fraction (1 + 2 + 3)	NA	0.56 ± 0.04	0.59 ± 0.05
	DA	0.95 ± 0.06	0.95 ± 0.08

[1]P of difference > 0.1. The two groups of treatment were not run at the same time. Test of significance of the difference between DA fraction 1, Table 5, estradiol treated and DA fraction 1, Table 3, hormone treated: $P > 0.1$.

producing the dissections exactly from experiment to experiment. As controls and experimentals were always run at the same time (except in Table 4 and 5) this possible inconsistency affects both treated and not hormone treated alike.

TABLE 6.
The cerebral serotonin content in spayed rats after treatment with estradiol benzoate, 50 μg/kg s.c. followed by progesterone, 0.4 mg/animal s.c. 48 hours later and Mo 911, 50 mg/kg i.v., at 52 hours. The animals were sacrified one hour after Mo 911 treatment. Each replicate (5) consists of tissue from 3 brains. The values are means ± standard errors of the mean, expressed in μg/g brain.

DISSECTION METHOD A:	Hormone treated	Controls
Fraction 1	1.31 ± 0.06	1.35 ± 0.04
Fraction 2	0.79 ± 0.05	0.79 ± 0.04

An attempt was made to determine a possible effect on the turnover of brain serotonin by estrogen-progesterone treatment. The monoamine oxidase inhibitor Mo 911 was injected 50 mg/kg intravenously and the animals were sacrified one hour later. At this time the decreased monoamine break down had resulted in an accumulation of serotonin of about 60—70 % (Compare Table 2 and 6). This response is within submaximal limits. The serotonin increase was the same whether the hormones had been administered or not (Table 6).

Discussion

The effect of increased cerebral monoamine levels

The recently described inhibitory effect on the estradiol-progesterone activated estrus behaviour by monoamine oxidase inhibitors (Meyerson, 1964 a) has been given added weight by the results of the present investigation with the monoamine oxidase inhibitor Mo 911. The investigation shows that the decreased heat response is very likely due to the increase of cerebral serotonin levels.

The relationship between decreased heat response after Mo 911 treatment and increasing levels of serotonin and noradrenaline is evident (Fig. 3). The dopamine level was however not changed during the increasing heat inhibitory phase, which could be seen by comparison of the 1 hour and 4 hour values in Fig. 3.

A comparison of the serotonin, dopamine and noradrenaline levels at 4 hours after Mo 911 treatment, i. e. when heat inhibition is very obvious, with the more selective increase of the amines after Mo 911 in combination with the amine precursors indicate that it is the serotonin increase which interferes rather than that of dopamine or noradrenaline (fig. 4). This seems true even if a delay in the release to the effector cell of freshly accumulated catecholamines could possibly exist when the increase is caused by means of exogenous precursors. Such a delay is suggested by the fact that the animals' general behaviour was very little changed at 1 hour after Mo 911 + precursor treatment (see also Meyerson, 1964 a, Table VII) in spite of high amine levels. However, it has been shown in earlier work (Meyerson, 1964 a) as well as in the present investigation that the heat inhibition observed 4—6 hours after Mo 911 is not increased if DOPA or DOPS are administered with the Mo 911. At such a long time after the injection the selectively increased catecholamines should be available at the receptor sites. Thus, there was no demonstrable effect on the heat response of acutely increased or more prolonged increased availability of catecholamines.

It might be said that an inhibitory effect on the heat response of catecholamines could not be fully excluded by merely correlating the heat inhibition with the brain amine levels (Fig. 3). For instance the unaltered dopamine level during the increasing heat inhibitory phase after Mo 911 may be due to an increasing release to the effector cells, and subsequent degradation by catechol-O-methyl transferase (COMT).

The presence of COMT in the brain was demonstrated by Axelrod (1958). Accumulating evidence supports its physiological significance. The 3-O-methylated catecholamines, normetanephrine and 3-methoxytyramine, occur normally in the brain as was shown by Häggendahl (1963) and disappeared after treatment with COMT-inhibiting substances. The possibility that the monoamine oxidase enzyme is close to the site of synthesis and that COMT occurs extracellularly near the effector cell was discussed by Carlsson (1960, 1964). It is suggested by the rapid accumulation of cerebral noradrenaline after administration of monoamine oxidase inhibitor in contrast to the rise in normethanephrine which follows some hours later. (Carlsson, Lindqvist and Magnusson, 1960). Similarly, when L-DOPA is injected into rabbits intravenously 3,4-dihydroxyphenyl acetic acid is accumulated and only later homovanillic acid (Carlsson and Hillarp, 1962).

Alpha-substituted dopacetamid derivatives have recently been described as potent COMT inhibitors (Carlsson, Corrodi and Waldeck 1963). The inhibitory effect on the heat response of Mo 911 was decreased when this treatment was combined with the COMT inhibitor H 22/54. This substance should bring about increased availability of catecholamines at postsynaptic receptors. The simultaneous decrease in catecholamine synthesis by phenylalanine and tyrosine hydroxylase inhibition seem to be of less importance. The dopamine and noradrenaline levels were nearly the same after Mo 911 + H 22/54 as after Mo 911 alone. Thus, the results indicate that the reduced estrus response after Mo 911 is not due to decreased degradation of *catecholamines*. If an increase in catecholamines were heat inhibitory, H 22/54 should not have counteracted Mo 911.

The decreased effect on the heat response of Mo 911 when combined with H 22/54 is probably due to inhibition of the *serotonin* synthesis by the inhibitory effect of H 22/54 on tryptophane hydroxylase (Carlsson, Corrodi and Waldeck, 1963).

The effect of amine depletion

In a previous paper the possibility was discussed that amine depletors (reserpine, tetrabenazine) removed monoaminergic heat inhibition (Meyerson, 1964 b).

The fact that estrus behaviour was not activated in the absence of estrogen administration might indicate that exciting activity mediating estrus behaviour does not exist continuously but only after the estrogenic hormone has been in action.

The present investigation shows that if the dose of estrogen is increased, less amine depletion is required to elicit the estrus response (Fig. 6, 7 and 8). The more excitation, the less heat inhibition needs to be removed.

The fact that a low degree of depletion is sufficient to elicit the heat response indicates that either the relevant structures have to be only partially depleted of transmitter or that they constitute a part of the brain especially sensitive to reserpine.

The fact that the effect of a single dose lasts for several weeks indicates that the relevant structures recover very slowly. As shown by Carlsson, Falck and Hillarp (1962) and Dahlström and Fuxe (1964) using a histochemical fluorescence method for demonstration of monoamines, the terminals are more easily depleted than the cellbodies and recover more slowly. The data from the work of Carlsson et al. concern catecholamine terminals. However, the said is also true for serotonin terminals (Fuxe, 1964). Within the suprachiasmatic nucleus the serotoninergic fibers stands out very distinctly. The yellow fluorescence characteristic for serotonin was very much reduced within this region even 10 days after 2 mg kg reserpine in ovariectomised rats treated in addition to the reserpine treatment with 25 µg kg estradiol 56 hours before they were sacrificed (Dahlström, Fuxe and Meyerson, unpublished data). It seems reasonable therefore to conclude that the observed removal of heat inhibition is due to the effect on terminals.

The effect of hormone treatment on brain amine content

It could be concluded from the results of the present investigation that an estradiol + progesterone treatment which normally activates estrus behaviour, did not bring about changes of cerebral serotonin, dopamine or noradrenaline levels compared with untreated controls. Nor were there any statistically significant changes in the amine levels when the hormones were given separately.

However, the results do of course not preclude the possibility of highly localised quantitative changes not detected by the method used.

Estrus behaviour and monoamines

Summary and Conclusions

[The following summary is based upon the present (III) and two earlier papers: Meyerson, 1964 a (I) and 1964 b (II).]

If the present and earlier data are taken together, there is good evidence for the assumption that in the female rat there exist monoaminergic pathways which mediate heat inhibition.

An increase in 'monoaminergic tone' would activate heat inhibition while a decrease would remove such an inhibition.

A. A general increase of the monoamine levels in the brain by different monoamine oxidase inhibitors (Mo 911, Nialamide, JB 516) brought about decreased estrogen-progesterone activated heat response (I). A more selective accumulation of serotonin, dopamine or noradrenaline achieved by precursor treatment revealed an obvious inhibition by increased serotonin levels while the catecholamine increase had no apparent effect (I and III).

Supporting the view that the effect of the monoamine oxidase inhibitors is due to the increased levels of serotonin is the fact that the effect of Mo 911 was less when combined with the tryptophane-hydroxylase inhibitor H 22/54 (III).

Even if there is good evidence that increased levels of serotonin bring about the heat inhibition the existence of special serotoninergic heat inhibitory receptors is still uncertain.

Serotonin might conceivably act at dopaminergic or noradrenergic receptors with competitive blocking of a heat stimulatory response.

Against this possibility speak the following findings: Heat inhibition at 2 or 4 hours after injection of Mo 911 alone was almost the same as in the experiments where DOPS (III) or DOPA (I) was injected as well. In these later cases greatly increased amounts of noradrenaline and dopamine were available.

Nor seems monoaminergic heat stimulation likely in regard of the effect of reserpine on estrus behaviour (see below).

B. Progesterone could be replaced by amine depletion caused by reserpine or tetrabenazine (II).

The effect of reserpine was not prevented by adrenalectomy. This excludes the possibility that is was caused by the secretion of progestative substances from the adrenals due to reserpine released ACTH (II).

The results from dose-response experiments indicate that the effect of reserpine might be at the terminal part of the axon (III); a response was elicited by a dose level reportedly insufficient to give a demonstrable effect on the nerve cell bodies, and a response was achieved a long time after a single dose of reserpine (II), indicating slow recovery which is more characteristic for the nerve terminals than the nerve cell bodies (cf. Carlsson et al. 1962, Dahlström and Fuxe, 1964).

It must be pointed out, that the discussion and conclusions above are partly based upon analyses of the amine content of extensive regions of the brain. This might be risky in view of the accumulating evidence that amines are stored within the neuron in various pools with different functional significance (see Kopin, 1964) and also considering the possibility of highly localized changes within certain areas or nuclei.

C. The inhibition of the estrogen-progesterone activated heat response was very evident after α-methylDOPA administration (I). The effect of α-methylDOPA was shown very probably to be due to decarboxylated metabolites as the effect was reduced by pretreatment with decarboxylase inhibitors. Considering the heat facilitating effect of reserpine (II), the improved heat inhibition of α-methylDOPA in animals pretreated with reserpine (I), the absence of inhibitory effects of decarboxylase inhibitors (I) and of hydroxylase-COMT inhibitors (H 22/54, unpublished observation), it seems possible to conclude that the effect of α-methylDOPA is not due to amine synthesis inhibition or displacement of endogenous amines. A direct effect of α-methylDOPA metabolites on heat inhibitory receptors must be considered.

DL-amphetamine reduced the estrogen-progesterone activated heat response (I). Vane (1960) demonstrated that D-amphetamine acts on tryptamine receptors in the rat stomach and Innes (1963) could extend this conclusions to guinea pig ileum, dog retractor penis, rabbit aorta and rabbit uterus. It therefore seems possible that amphetamine and perhaps also α-methylDOPA-metabolites are able to stimulate central nervous serotoninergic heat inhibitory receptors.

As an indicator that the animals are in heat the lordosis response stimulated by mounting has been used throughout the investigation. Also other manifestations of estrus behaviour were activated by reserpine in estrogen pretreated spayed rats (III). It therefore seems probable that the monoaminergic heat inhibition includes other manifestations of heat in addition to the lordosis response.

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